STUDY ON PREVALENCE OF ENTERIC PARASITIC INFECTIONS IN HIV-INFECTED PATIENTS IN CHENNAI

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CERTIFICATE

This is to certify that this dissertation entitled "**STUDY ON PREVALENCE OF ENTERIC PARASITIC INFECTIONS IN HIV-INFECTED PATIENTS IN CHENNAI"** is the bonafide work done by **Dr. ANAND B. JANAGOND** in the Department of Microbiology, Stanley Medical College, Chennai in partial fulfillment of the regulations laid down by the Tamil Nadu Dr.M.G.R.Medical University, Chennai for M.D. Microbiology (Branch IV) examination to be held in September 2009.

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I solemnly declare that this dissertation "**STUDY ON PREVALENCE OF ENTERIC PARASITIC INFECTIONS IN HIV-INFECTED PATIENTS IN CHENNAI"** is the bonafide work done by me at the Department of Microbiology, Government Stanley Medical College and Hospital, Chennai, under the guidance and supervision of **Prof. Dr. P. R. THENMOZHI VALLI, M.D.,** Professor and Head, Department of Microbiology, Government Stanley Medical College, Chennai-600 001.

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CONTENTS

INTRODUCTION

Human immunodeficiency virus (HIV) infection is a global pandemic and an extremely serious worldwide problem.[31] According to the 2006 report on the global AIDS pandemic by UNAIDS, the total number of persons living with HIV is 39.5 million .[65]

India is on the verge of having the greatest increase in the estimated number of people living with HIV/AIDS (PLHA) in the world in the coming decades.[1] Nationally, the HIV prevalence among adults (aged 15 – 49 years) is less than 1%, but with a population of more than 100 crores, estimated population of PLHA is 51.34 lakhs by the end of 2004 (NACO 2005 b).[53] According to the UNAIDS statistics there were estimated 1,60,000 AIDS cases in India in 2006.[65] India is second only to South Africa in terms of the overall number of people living with the disease.[52]

In some north-eastern states and southern states of India, including Tamil Nadu, the HIV epidemic is classified as a generalized one, with more than 1% of women attending antenatal clinics (ANCs) being infected and an HIV prevalence among sexually transmitted infection (STI) clinic patients of more than 5%. The first AIDS case in India was discovered in a female commercial sex worker in Tamil Nadu in 1986.[53] According to the UNAIDS' statistics there were estimated 58,000 AIDS cases in Tamil Nadu in 2006; HIV infection rates in STD patients being 9.2%.[52]

HIV infection leads to acquired immunodeficiency syndrome (AIDS) and major cause of morbidity and mortality of such patients are opportunistic infections.[5] With the immune system being defective, these individuals have an abnormally high susceptibility to

infections with non-virulent minimally pathogenic organisms[6]. Many of these individuals contract parasitic infections in addition to suffering numerous infectious episodes with bacterial, viral and fungal organisms.[39]

The association between the selected enteric parasites and HIV infection is well documented. For example, the intracellular intestinal protozoan parasites *Cryptosporidium parvum, Cyclospora cayetanensis, Isospora belli* and the *Microsporidia* are often the major cause of uncontrollable, debilitating diarrhea. These parasites cause high morbidity and mortality in AIDS patients worldwide and these outcomes would be expected to be appreciably higher in developing countries due to higher prevalence of infections in the general population. Little, however, has been reported on the presence of other protozoan and helminths infections in HIV positive individuals in developing countries.[38]

Based on high prevalence, non-opportunistic parasites may also be major contributors to morbidity in patients already compromised by AIDS living in developing countries.[38] Similar to opportunistic parasitic infections, diarrhea is a common clinical symptom associated with infection by non-opportunistic parasites[38].

Collectively, parasites cause diarrhea in almost 90% patients with HIV in developing countries.[3,35] It is associated with weight loss and is often the presenting illness of HIV-infected individuals. This diarrhea, wasting syndrome, in association with positive HIV serology test, is an AIDS defining illness in WHO's classification.[53]

The type of pathogen responsible for morbidity and mortality vary from region to region and it also varies with time. Therefore, identification of specific pathogen(s) is important for management of such cases.[2] Infections caused by these parasites can not be

differentiated clinically unless specific faecal examination is carried out.[56] Since the enteric illness due to parasitic aetiology among HIV patients is on the rise during recent times, the present study was undertaken to study the status among HIV patients in Chennai.

AIMS AND OBJECTIVES

1. To identify parasites causing intestinal infection in HIV seropositive patients.
2. To compare various methods for detection of intestinal parasites.
3. To estimate the prevalence of opportunistic intestinal parasites in HIV infected patients in Chennai.
4. To correlate opportunistic intestinal parasitic infections with immune status of the patients (CD4 T lymphocyte counts).
5. To correlate opportunistic intestinal parasitic infections with duration of diarrhoea.

REVIEW OF LITERATURE

AIDS was recognized for the first time in June, 1981 at the Center for Disease Control, USA.[23] HIV, the virus causing AIDS, was independently identified by a team of French scientists led by Dr. Luc Montaignier of Pasteur Institute of Paris and American Scientists led by Dr. Robert D Gallo. Now, HIV/AIDS has become a global health problem.[52]

CD4 cells are the main targets of HIV and progressive destruction of these cells is characteristic of all stages of HIV disease leading to immunocompromised status in the affected individual.

Mechanism of CD4 cell depletion and dysfunction in HIV infection[24]

HIV can kill CD4 cells singly or after giant cell and syncitial formation. Single cell killing occurs due to accumulation of unintegrated viral DNA and inhibition of cellular protein synthesis. Syncitium formation is induced by virulent strains of HIV in a multistep mechanism. CD4 cells expressing viral antigens on the surface attract CD4 uninfected cells and the membranes of these fuse producing giant cells and syncitia. One such HIV infected cell can eliminate hundreds of uninfected cell by syncitium formation.

The non-virologic mechanisms which can damage /destroy CD4 cells include autoimmune mechanisms, anergy, superantigens, apoptosis (programmed cell death) and virus specific immune responses.

Diagnosis of HIV infection [22, 23]

Detection of anti-HIV antibodies is the mainstay of testing for HIV and diagnosis of HIV. Tests to detect specific HIV antibodies can be classified into Screening tests (ELISA/EIA and Rapid) and Supplemental tests (ELISA/EIA and Rapid and Western Blot). Screening tests are performed to screen units of donated blood and blood products and for surveillance. Supplemental tests are performed on serum sample reactive in screening test for the purpose of diagnosis of the individual. If a specimen is reactive in two different systems, it has to be tested again using one of the supplemental tests which may be a third ELISA/Rapid test or a Western Blot test (WB) as the case may be. Which ever commercial kit is selected, it should be ensured that it detects antibodies against HIV-1, HIV-2 and their subtypes. Strategies devised by UNAIDS and WHO which are based on clinical presentation of the individual and prevalence of HIV have to be followed.

Laboratory Markers Associated with Progression of HIV Infection

The risk of opportunistic infections is known to increase with increasing immunodeficiency. The laboratory markers available for assessing immune status of HIV infected patients can be classified into:

1.Viral Markers

i. Quantitative Viral Load – It can be estimated by assaying, Plasma HIV Viral RNA Load, Serum p24 Antigen or Serum p24 Antibody.

ii.Assays for Qualitative Viral Change – For example Syncitium Inducing Strains, which are more virulent.

2.Surrogate Markers

I. Virus specific markers - Decline in, or absence of antibodies to various HIV antigens including p24, p17, gp120, gp41 and nef gene product have been used as surrogate markers in the past, but they are not very sensitive.

II. Non-specific markers

A. Cellular Markers / Markers of Immune Function – These include CD4 cell count, Percentage of CD4 cells, CD8 cell count, Multitest, delayed type hypersensitivity (DTH) skin test and Phenotypic markers of lymphocytic activation.

B. Soluble Markers – For example Neopterin and Beta 2-microglobulin (β2-m).

C. Other Markers - Other surrogate markers of immune function, such as soluble interleukin-2 receptor levels, serum IgA levels and serum cytokine levels have been studied. Other potentially promising immunologic prognostic markers include percentage of CD38, CD4 cells and CD4, CD29 "bright" memory cells.

CD4 T lymphocyte Count [52,36]

CD4, a surface glycoprotein on certain T cells, serves as a receptor

for HIV, and cells expressing this protein usually decline in number with progressive HIV infection. The number of cells that express the CD4 antigen is therefore a usual guide to the pathological effects of HIV on the immune system. Studies have shown that subjects with low CD4 count are at risk of specific AIDS related illnesses such as various opportunistic infections. Several technologies for determining the absolute number of CD4 cell counts have been developed. These are

Flow cytometric method – Immunofluorescence analysis by flow cytometry is the gold standard for CD4 T lymphocytes measurements and also the method of choice if a large throughput of samples is required. To date, many single-platform flow cytometric technologies have been developed commercially, some of them are - FACSCount microbead-based system, Modified flow cytometry, Guava EasyCD4 volumetrc system, Partec CyFlow counter (volumetric system) and PointCARE system.

Non- flow cytometric methods – Manual methods - Microscope based, low cost, microbead separation of CD4 T lymphocytes from other blood cells, followed by standard manual cell counting techniques using a light microscope. Dynabeads CD4 T lymphocytes quantitation is another example.

Diarrhoea and Intestinal Parasites in HIV/AIDS

Infections by opportunistic pathogens including various types of

intestinal parasitosis have been the hallmark of AIDS since the beginning of the epidemic. Diarrhoea is the major gastrointestinal symptom in HIV infection affecting 90% of the patients, and it becomes more frequent as immunodeficiency progresses. Diarrhoea and weight loss are independent predictors of mortality.[57] These infections are expected to be appreciably higher in developing countries due to higher prevalence of infections in general population.[70]

Epidemiology of Intesinal Parasitic Infections in HIV

Various studies have shown the high prevalence of intestinal parasites in HIV-infected individuals and the varying trends with time and place.

In 1999, Mukhopadhya, Ashis, Ramakrishna *et al*. conducted study to determine the carriage rate of various enteric pathogens in 111 HIV seropositive patients both with (61) and without (50) diarrhoea. Intestinal parasites were present in 57.4% of patients with diarrhoea and in 40% without diarrhoea. Protozoa accounted for 71.8% of the stool samples tested. *Giardia intestinalis* was found in 14.9% with diarrhoea and in 32% without diarrhoea, *Isospora belli* in 18% with diarrhoea and 8% without diarrhea and *Cryptosporidium* in 10% of both the diarrhoeal and non-diarrhoeal samples. Addition of jejunal fluid examination and biopsy increased the total yield of pathogens to 70% in acute diarrhoea and 80.6% in chronic diarrhea.[48] In 2000, Prasad KN, Nag VL, Dhole TN *et al*. conducted a study to identify intestinal parasites in 59 HIV-positive

patients with diarrhoea. Intestinal parasites were found in 73% of patients. Of them 89% harboured single parasite and 11% of patients had mixed parasites. *Isospora belli* was detected in 30.7%, *Cryptosporidium* 15.3%, *Blastocystis hominis* 7.7%, Strongyloides species 3.8%, *Entamoeba histolytica* 11.5% and *Giardia lamblia* in 3.8%.[56]

In 2002, Satheesh KS, Ananthan S, Lakshmi P conducted study on 150 HIV-seropositive stool samples (41-acute diarrhoea, 59 chronic diarrhoea and 50 without diarrhoea). Intestinal parasites were detected in 39% patients with diarrhoea and 14% patients without diarrhoea. *Isospora belli* was commonest in 18%, followed by *Cryptosporidium* 14%, *Cyclospora* 4% and *Microsporidium* 8%. Among controls, *Cryptosporidium* in 8%, Hookworm 4%, *Entamoeba histolytica* and Strongyloides species 2% and *H. nana* 0.6%.[35]

In 2004, Zali MR, Mehr AJ, Rezaian M *et al*. conducted a study on 206 stool samples of HIV-seropositives. The overall prevalence of intestinal parasites was 18.4%, *Giardia lamblia* in 7.3%, *Blastocystic hominis* in 4.4%, *Entamoeba coli* in 3.9%, *Cryptosporidium* in 1.5%, Strongyloides species and *Hymenolepsis nana* in 0.9% each. CD_4 counts were significantly lower in individuals with diarrhoea than those without diarrhea.[70]

In 2007, Ramakrishnan K, Shenbagarathai R, Uma A *et al*. studied on 80 HIV / AIDS patients and 80 HIV seronegatives at Madurai city.

Overall parasitic infection rate was 38.7% and 17.5% respectively. In HIV seropositives, *Cryptosporidium* was commonest in 28.7%, followed by *Entamoeba histolytica* 17.5%, *Entamoeba coli* 20%, *Ascaris lumbricoides* 7.5%, *H. nana* 1.2%, *Giardia lamblia* 3.7% and *Isospora belli* 1.2%[57].

Parasitic infections that cause self-limited diarrhoea in immunocompetent patients may cause profuse diarrhoea in immunocompromised individuals. *Isospora belli, Cryptoporidia, Microspridia, Cyclospora* have been shown to cause more common and severe infections in HIV patients.[6]

Isospora belli

Isospora belli was described for the first time in humans in 1915 by Woodcock and later by Wenyon in 1923.[32] The name 'belli' was given because several cases of infection with this parasite were seen among troops stationed at the Middle East during the first world war (Bellium meaning war).[16]

Human *I.belli* infections differ from those of other coccidians in that both the sexual and asexual forms inhabit the human intestine and a second host is not required to complete the life cycle. The disease is not a zoonosis.[34] Intestinal pathology with blunting of the villi, villous atrophy, and collagen deposition in the lamina propria is commonly seen.

I.belli infects both immunocompetent and immunocompromised

adults and children, symptoms range from none to diarrhoea. Persistent non-bloody diarrhoea, similar to that seen with microsporidiosis and cryptosporidiosis, is the major symptom seen in immunocompromised individuals.[39]

Parasitic diagnosis is made by demonstration oocysts in feces or in duodenal samples. Typical large elliptical-shaped *I.belli* oocysts can be detected in the direct wet mounts of stool and also by using bright field, differential interference contrast microscopy. Oocysts have the property to autofluoresce under ultraviolet rays. This can be demonstrated by using 450-490 excitation filter.[68]

In acid-fast staining of stool smears the pink colored acid-fat oocysts can be demonstrated in stool smears stained by modified acid-fast staining method. The stool smears can also be stained by auramine-rhodamine, hematoxylin and eosin, and Giemsa stain.

Cryptosporidium Spp

Cryptosporidiosis a disease caused by *Cryptosporidium sp*. was described for the first time in 1907 by Tyzzer. This parasite was considered a commensal up to 1975, when it was identified as the cause of diarrhoea in animals.[8] The first manifestation of cryptosporidiosis was reported by Nine *et al*. in 1976, and the disease became a major concern when the

notification of the first 21 patients was given, 14 of whom died of chronic diarrhoea caused by Cryptosporidial infection.[10]

Cryptosporidium hominis has been implicated as the major cause of cryptosporidiosis in humans in most areas. Other species that have been found in humans include *C.parvum, C.meliagridis, C.feli, C.canis, C.muris, C.suis* and Cryptosporidium corvine genotype. Among animal hosts calve are the most important from the public health point of view, as they may serve as a source of human infection.[14] Oocysts are not eliminated by chlorination .[68]

Oocysts have a propensity to adhere to the brush border of epithelial cells with loss or degeneration of microvilli at the attachment zone 196. The clinical syndrome of cryptosporidiosis includes a cholera-like watery or mucous diarrhoea, persistent gastroenteritis with varying degrees of vomiting and abdominal cramping, malabsorption and low grade fever.[34]

The diagnosis is commonly made by identifying oocysts in fecal specimens. They appear as red-staining spherical oocysts 4 to 6 um in diameter, with sporocysts, when observed in acid-fast stained stool preparations. Other staining methods that can be employed are the safranine methylene blue stain, the auramine phenol fluorescent stain, the auramine carbol fuchsin fluorescent stain, the mepacrine potassium permanganate stain, etc. [30]

Among persons with profuse diarrhoeal illness, a single stool specimen is usually adequate for diagnosis.[24] Formalin-ethyl acetate technique and Sheather's sugar floatation technique are recommended as concentration procedures in lighter infections.[34]

Several immunoassay kits are commercially available for the detection of Cryptosporidium Oocyst in fecal specimens. These have been found to have sensitivity of 98-99% and a specificity of 100%.[34] Several extremely sensitive and specific PCR-based methods are also available.[68]

Cyclospora cayetanensis

The first published report of *Cyclospora cayetanensis* in humans was by Ashford in 1979, who found it in faeces of three individuals in papua, New Guinea. It was thought to resemble *Cyanobacteria* and the name *Cyanobacterium*- like body or "*CLB*" became prevalent in literature.[27]

In 1994, Ortega, Gilman and Sterling identified the parasite and named it *Cyclospora cayetanensis*. The organism was first identified by microscopy in 1998 and by PCR in 1999.

Humans acquire infection by ingestion of water or vegetables contaminated with sporulating oocysts. The oocysts undergo sporulation outside the host before becoming infective. Small intestine shows acute and chronic inflammation, blunting and atrophy of villi, and hyperplasia of crypt. Diarrhoea is the key clinical feature in patients with cyclosporiasis.[39]

Diagnosis rests on the microscopic detection of oocysts in fecal specimens in direct wet mount or by acid-fast staining methods. Oocysts are variably acid-fast. In a positive stool smear, approximately 50% of the oocysts are acid-fast and pink coloured and the rest are non-acid-fast and colorless.[54]

Microsporidium spp.

In 1977, the class or order *Microsporidia* were recognized in mammalian tissue samples, and were suspected as being cause of human disease when it was detected in a child with encephalitis.[61]

Microsporidia are obligate intracellular and spore forming parasites. They show following unique features. They possess resistant spores and they possess a polar tubule or polar filament, a unique organelle, present inside the spore.[39]

There are at least 13 species belonging to the genera *Enterocytozoon, Encephalitozoon, Nosema* and *Pleistophora,* which are known to cause human disease. Most cases (90%) of intestinal microsporidiosis are caused by *Enterocytozoon bieneusi* and the rest are caused by *E. intestinalis.*[39]

Spores are the infective forms of the parasite. Humans acquire infection by ingestion or inhalation of the spores. Microsporidia cause protracted debilitating chronic diarrhoea, which may occur for several months, and malaborption and wasting. The mortality due to diarrhoea in these patients is as high as 56%. *Microsporidia* can cause lesions in eye,

musculo-skeletal system and also disseminated infection.[68]

The modified trichrome stain (chromotrope 2R), rapid Gram chromotrope method and fluorochrome stains including Uvitex 2B and calcoflour white are commonly used methods to stain microsporidia in stool, urine and other body fluids. Direct fluorescent method using monoclonal antibodies are available. Transmission electron microscopy is useful in species identification of microsporidia, based on the ultra structure. PCR is available to detect *E.beneusi, V.corneae* and *Nosema* species. [68]

Strongyloides stercoralis

The life cycle of *Strongyloides stercoralis* is similar to that of hookworms except that the majority of the eggs hatch into rhabditiform larvae while still in the intestinal lumen. In patients who have lost immunity rhabditiform larvae may next hatch into filariform larvae within the intestinal lumen, from which they can directly invade the mucosa resulting in disseminated reinfection disease. Humans get infected by direct penetration of filariform larvae into the skin on direct contact.[34]

Immunosuppressed hosts are particularly vulnerable to disseminated Strongyloides infections. The propensity for *S. stercoralis* eggs to hatch quickly and produce intraintesinal filariform larvae makes patients vulnerable to auto infection, producing a condition known as hyperinfection syndrome.[54]

Diagnosis is achieved by demonstration of larvae in stool specimen. The concentration procedure of Baermann, Formalin-ethyl acetate method, Agar plate method, Lutz method and Harada-Mori culture method may be employed to detect larvae in stool specimen with lighter load.[5]

Characteristics of larvae of *Strongyloides stercoralis* and Ankylostoma duodenale or Necator americanus [40]

Larval stage	*S. stercoralis*	*A.duodenale /N.americanus*
Rhabditiform	1.Buccal cavity short (4 um) 2.Esophagus one-third of body length with 2 swellings 3.Genital primordium large (22 um) 4.Anal pore 50 um from posterior end	1.Buccal cavity long (15 um) 2.Esophagus one-third of body length with 2 swellings 3.Genital primordium small (7 um) 4.Anal pore 80 um from posterior end
Filariform	1.Size 200-500 ×15-20 um 2.Unsheathed 3.Tail forked or blunt 4.Esophagus half of body length with no swelling	1.Size 200-500 ×14-20 um 2.Sheathed 3.Tail tapered 4.Esophagus one-third of body length with no swelling

Diagnosis of Intestinal parasites

Faeces is the most commonly used specimen for detecting intestinal parasites. Ideally, three specimens are collected, one every other day or within a 10-day time period.[39]

Microscopic examination[39,54]

Wet Mounts:

Wet mounting is the simplest and easiest technique for the examination of feces. A wet mount can be prepared directly from fecal material or from concentrated specimens.

Saline wet mount

The direct saline wet mount is used primarily to detect motile protozoan trophozoites. Helminth eggs and/or larvae, protozoan cysts and coccidian oocysts can also be seen. One disadvantage of saline wet mounts is that the inner details of the parasites are not visualized distinctly.

Iodine wet mount

Dobell and O'Connor's, Lugol's and D'Antonie's iodine are different types of iodine frequently used in the iodine wet mount. Iodine wet mount is mainly used for protozoal cysts. The chromatoid bodies of protozoan cysts are not clearly visible in iodine mounts. The motility of trophozoites is inhibited in the iodine wet mount.

Lactophenol Cotton Blue (LPCB) wet mount

The LPCB is a common reagent used for microscopic identification of fungi. The LPCB is a combined fixative, staining and clearing agent. The LPCB stains internal structure of trophozoites, cysts and ova, thus facilitating their recognition and identification in stool specimens. It contains phenol and lactic acid which clear the fecal debris. Glycerol in

LPCB prevents drying of the wet mounts and makes the preparation semi permanent.[54]

LPCB fecal mounts have certain advantages over routinely employed saline and iodine mounts. LPCB fecal mounts provide stained smears which can be easily prepared and preserved. The stained smear is important for providing complete and accurate examination of intestinal parasites. Application of LPCB fecal mounts for routine parasitic examinations along with saline and iodine can increase the sensitivity.[47]

The motility of protozoan trophozoites and helminthic larvae is lost instantly in LPCB mounts. It also causes shrinkage, destruction and lysis of trophozoites, pus cells and red blood cells enabling them difficult to identify. LPCB stains all fecal components bluish including undigested food debris, although parasitic elements can be distinguished from them by a well defined outer layer (cyst wall and egg shell) which in fact becomes more prominent with slight shrinkage of internal structures of cyst and ovum.[47]

Concentration Methods[39]

Concentration methods allow the detection of small number of organisms that may be missed by using only a direct wet smear. There are two types of concentration procedures, both of which are designed to separate protozoan organisms and helminth eggs and larvae from fecal debris by centrifugation and/or differences in specific gravity.

1. **Floatation Techniques** - Saturated salt solution method, Zinc sulphate floatation method, saturated magnesium sulphate solution method and sugar floatation methods are some of the examples.

2. **Sedimentation Techniques** - Sedimentation methods (centrifugation) lead to the recovery of all protozoa, oocysts, eggs, and larvae present; however, the concentrated sediment that is examined contains more debris. If one technique is selected for routine use, the sedimentation procedure is recommended as being the easiest to perform and the least subject to technical error.

Formalin ether sedimentation method, Formalin ethyl acetate sedimentation method, Simple gravity sedimentation methods are some of the examples for concentration methods by sedimentation. Automated workstation for the Microscopic Analysis of Fecal Concentrates are also available (FE-2 - DiaSys Corp., Waterbury, Conn.)

Permanent Stained Smears[39]

The detection and correct identification of many intestinal protozoa frequently depend on the examination of the permanent stained smear with the oil immersion lens (100X objective). These slides not only provide the microscopist with permanent record of protozoan organisms identified but also may be used for consultations with specialist when unusual morphologic characteristics are found.

Examples for Permanent Staining Methods are - Trichrome Stain, Iron Hematoxylin stain, Modified Iron Hematoxylin stain, Polychrome IV Stain, Chlorazol Black E Stain etc. There are specialized Stains for Coccidia and Microsporidia like Modified Kinyoun's Acid-Fast Stain, Modified Ziehl-Neelsen Acid-Fast Stain, Carbol Fuchsin Negative Stain for Cryptosporidium, Rapid Safranin Method for Cryptosporidium, Rapid Safranin Method for Cyclospora using Microwave, Auramine O Stain for Coccidia, Modified Trichrome stain for Microsporidia, Acid-Fast Trichrome Stain for Cryptosporidium and Microsporidia etc.[39]

MATERIALS AND METHODS

Type of study - A descriptive cross sectional study design was used.

Study Population

A total of 150 individuals were included in the study after the approval of institutional ethical committee and with the consent of the subjects.

Cases: 100 randomly selected patients, seropositive for HIV 1 and/or 2, attending Govt. Hospital of Thoracic Medicine, Thambaram Sanatorium and Govt. Stanley Hospital, chennai as inpatients or outpatients were included in the study. Cases included patients with and without diarrhea.

Inclusion criteria - Seropositive for HIV 1 or HIV 2 or both

1. With or without diarrhea.
2. All age groups and gender

Exclusion criteria - Patients who had received Anti-parasitic medications, anti-diarrheal agents in the previous 2 weeks [48,15].

Controls: A total of 50 patients attending Govt. Stanley Hospital not having symptoms and signs of HIV infection were included in control group.

Period of study - October 2007 to November 2008

Sample collection

At least 2 freshly passed stool specimens were collected on consecutive days in wide mouthed, leak proof, plastic containers without any

preservative.[8] A disposable plastic spoon was kept inside each container for the convenience while collecting the sample. Patients were instructed to drop the spoon along with the sample inside the container and close the lid tight. The containers were labeled properly and transported to the laboratory. The specimens were processed within 1-3 hours of collection.[39]

Sample Processing

Gross examination was done for all the samples to note colour, consistency, presence of blood / pus/mucous, adult worms or segments.

Microscopic Examination

Direct microscopic examination of feces in saline, iodine and lacto-phenol cotton blue suspension was done to detect trophozoites, ova, cysts, larvae and oocysts.

1. **Saline Wet Mount[39]**
    - One drop of 0.85% NaCl was placed on a clean glass slide. A small amount of representative fecal specimen was picked up on the end of an applicator stick and emulsified thoroughly.
    - A 22X22 mm clean coverslip was carefully placed over the suspension avoiding air bubbles.
    - This was used for identifying various forms of intestinal parasites (motile trophozoites, ova, cysts, oocysts and larvae).

2. **Iodine Wet Mount**[39]

   Iodine wet Mount was prepared similar to Saline Wet Mount, using D'Antoni's[39] Iodine. This was mainly used for identifying cysts. 0va, oocysts and larvae of parasites were also visualized.

3. **Lacto-phenol Cotton Blue Wet Mount**[54]

   This was done similar to the above wet mounts using Lacto-phenol Cotton Blue solution.

After preparing the above wet mounts, the entire cover slip area was first screened systematically under low power (total magnification, 100 X), starting at one corner and following a zig-zag path till the opposite corner of the coverslip. If something suspicious was seen, the 40× objective was used for more detailed study. At least 1/3 of the coverslip area was examined under high dry power (total magnification, 400 X).[39]

The following **permanent staining methods** were used for detecting coccidian parasites in the faeces specimens. A small portion of the sample was placed at the center of a clean slide and a thin smear (one should be able to see through the wet material before it dries) was prepared by gently rolling an applicator stick to spread it. Smear was air dried and heat fixed.

1. **Modified Kinyoun's Acid-Fast Staining Method (Cold method)**[39]

   - Heat fixed faecal smear was covered with a strip of filter paper. The slide was flooded with Kinyoun's carbol fuchsin and allowed to stain for 5 min.

- The stain was tipped off and filter paper removed. The slide was rinsed briefly (3 to 5 sec.) with 50% ethanol and then thoroughly with water.
- 1% sulfuric acid was used to decolorize for 2 min or until no more colour ran from the slide. Slide was then rinsed in water and drained.
- Counterstaining was done with methylene blue for 1 min. Slide was rinsed with water and air dried.
- Then the slide was examined with low and then high dry objective. To see the detailed morphology, oil immersion objective was used (total magnification, X1000).

2. **Modified Trichrome Stain for the *Microsporidia*[39]**

- Smears were prepared by spreading specimen over an area 45 X 25 mm on a clean glass slide. Smear was air dried and placed in absolute methanol for 5 min for fixation.
- Smear was then air dried. Placed in Trichrome stain for 90 min.
- Rinsed in acid alcohol for no more than 10 s. Slide was dipped several times in 95% ethanol for 5 min to rinse.
- Placed in 95% ethanol for 5 min and then in 100% ethanol for 10 min. It was then placed in xylene for 10 min.
- After air drying, smear was examined under 1000X or more and read at least 300 fields, taking about 10 min per slide.

- Known positive control smears were used.

Parasites were identified using the following features.

Entamoeba histolytica/dispar [54,33]

Trophozoite

Size and Shape: 12-45 µm; when moving, elongated and changing; when not moving, round.

Motility: Moves in one direction; a pseudopodium pushes forward and endoplasm flows rapidly into it. Motility is seen in saline wet mounts only.

Cytoplasm: The ectoplasm is transparent and quite different from the fine granular texture of the endoplasm (grayish with yellowish-green streaks), which may contain vacuoles. In LPCB preparations, cytoplasm is stained deep blue with pseudopodium stained light blue; motility is inhibited. Nucleus: Not visible in the motile form, but when stained with iodine solution clearly seen to have a regular membrane and a small dense central karyosome. Details are well made out in LPCB preparations also.

The invasive forms are larger (20-35 µm) and may have vacuoles containing more or les digested erythrocytes (hematophagous). The non-invasive forms are smaller (12-20 µm) and do not show hematophagy.

Cyst

Size and Shape: 12-15 µm; round

Nuclei: 1-4 nuclei, better visualized in Iodine and LPCB mounts. Membrane is thin, regular and circular. Karyosome is small, compact and central.

Cytoplasm: yellowish-gray after staining with iodine solution, granular: “dirty

appearance". In LPCB mounts cytoplasm is stained deep blue with light blue, clearly defined cyst wall. Chromatoid bodies, if present, are oblong, rounded at ends (sausage shaped).

Vacuole: sometimes a large glycogen vacuole (stained reddish brown with iodine solution) in young cysts with one or two nuclei.

Entamoeba coli [54,33]

Trophozoite

Size and Shape:20-40 µm (usually bigger than E. histolytica);

Oval or elongated, rather irregular, often non-motile or moving very slowly, putting out blunt pseudopodia in all directions.

Cytoplasm: Both ectoplasm and endoplasm are granular and difficult to differentiate. LPCB stain cytoplasm deep blue. Cytoplasm may have numerous and varied inclusions (bacteria, yeast cells, cell debris), but never erythrocytes.

Nucleus: visible in the fresh state, without staining. The membrane is irregular and granular (like bead necklace), the karyosome large and eccentric. Details of nucleus are seen well in iodine and LPCB preparations.

Cyst

Size and Shape: 12-20 µm; round or slightly oval

Nuclei: 1-8 nuclei. Nuclear membrane is irregular, thick in parts, not a regular circle. Karyosome is large, diffuse, and often eccentric

Cytoplasm: pale yellow after staining with iodine solution bright as compared

with *E. histolytica*. Chromatoid bodies when present are sharp or jagged ends (dagger shaped or needle shaped); not found in all cysts

Vacuole: Sometimes a very large vacuole (stained reddish-brown by iodine solution) compressing two nuclei, one at either pole.

Giardia intestinalis [54,33]

The vegetative and cystic forms of Giardia intestinalis are often found together in soft stools

Trophozoite

Size and Shape: 10-18 µm; rather elongated. Front view - pear shaped: side view – spoon shaped.

Motility: either moves forward in mall rapid jerks in a definite direction, sometimes turning in a loop (fluid tools), or is hardly motile.

Nuclei: two large oval nuclei, faintly visible.

Cyst

Size and Shape: 8-12 µm; oval, one pole more rounded than the other

Shell: often appears to be thick with double wall; the second wall is the membrane of the cytoplasm. The cyst wall is surrounded by clear halo in wet mounts.

Nuclei: 2-4 oval nuclei (not clearly seen). Details of nuclei are better visualized in iodine and LPCB preparations. Nuclear membrane is very fine and karyosome is small, central, faintly coloured.

Cytoplasm: clear, shiny when unstained; pale yellowish-green or bluish after

staining with iodine solution. In LPCB preparations the cytoplasm is stained deep blue and the pace between cyst wall and the cytoplasm is stained light blue and is refractile.

Fibril: shiny, hair-like line, folded in two or S-shaped, placed lengthwise in the centre of the cyst. It is stained deep blue in LPCB mount.

Hook worm– Ova [54,33]

Size and Shape: 50-80 µm; oval with rounded with lightly flattened poles Shell is very thin, appear a black line.

Content: varies according to the degree of maturity

In fresh stools four, eight or 16 grey granular cells are seen, being clear but not shiny (blastomeres). In stools a few hours old, a uniform mass of many small grey granular cells are seen. In stools 12-48 hours old, the whole of the egg is filled by a small larva, wrapped around it. The egg is 'embryonate'.

In LPCB preparations, blastomeres are seen deep blue and the space between the eggshell and segmented ovum is stained light blue. The eggshell is not stained or very lightly stained but clearly discernible.

***Ascaris lumbricoides* – Ova** [54,33]

Fertilized egg

Size and Shape: 45-75 µm; oval or sometimes round.

Shell: In corticated egg two shells are distinct. The external shell is rough, brown (bile-stained) and covered with mall lump (mamillated).The internal shell is smooth, thick and colourless. In decorticated fertilized egg, shell is

single layered, smooth, thin and colourless.

Content: a single round granular central mass.

Colour: external shell – brown: content – colourless or pale yellow. In LPCB preparations, the outer corticated, thick wall is stained deep blue. The eggshell is stained lightly. The ovum is stained deep blue, while the polar space between the eggshell and the ovum is stained light blue.

Unfertilized egg

Size and Shape: 45-90 µm; more elongated than the fertilized (elliptical or irregular).

Shell:the two shells are indistinct. The external shell I brown and puffy, withrather jagged lumps. The internal hell is thin (one or two lines may be visible). In semi-decorticated un-fertilized eggs shell is single, smooth, thin and colourless.

Content:the egg is full of large, round, very shiny granules.

***Trichuris trichura* – Ova** [54,33]

Size and Shape: 50-60 µm; Barrel shaped

Shell: fairly thick and smooth, with two layers, orange colour

Content: a uniform granular mass; yellow colour

Other features: a rounded, transparent mucous plug at each pole. In LPCB preparations, the double eggshell is stained deep blue. Mucus plug are not stained or lightly stained and well-defined. The un-segmented ovum is deeply stained and distinctly differentiated from the eggshell.

***Strongyloides stercoralis* – larvae** [54,33,40]

Rhabditiform larvae - Buccal cavity is short (4 µm). Oesophagus is 1/3 of body length with two swellings. Genital primordium is large. Anal pore is 50 µm from posterior end.

Filariform larvae - Measures 200-500 µm X 15-20 µm, is unsheathed. Tail is forked or blunt. Oesophagus is half of body length with no swelling.

***Isospora belli* – oocysts** [54,39]

Size and shape: oocysts measure 20-30 µm X 10-19 µm; sporocysts measure 9-11µm. Oocysts are ellipsoidal with spherical sporocysts.

Other features: oocysts can be visualized in wet mount preparations but identification is improved with modified acid-fast staining. Immature oocysts have single sporocyst and the entire oocysts stain pink to red with modified acid-fast staining, while those that are mature appear with the two sporocysts within the oocyst wall stained pink to red and a clear area between the stained sporocysts and the oocyst wall.

***Cryptosporidium spp.* – oocysts** [54,39]

Size and shape: 4-6 µm, round. Because of their smaller size, cryptosporidium oocysts are difficult to demonstrate in wet mounts.

Stain pink to deep red with modified acid-fast stain. The sporozoites within the oocysts have an outer rim of deep stained material with a pale centre.

***Cyclospora cayetanensis* – oocysts** [54,39]

Size and shape: 8-10 µm, round

In wet mounts they appear as round structures with relatively clear interior.

In modified acid-fast stained smears, they show tremendous acid-fast variability (clear to pink to red to deep purple). Oocysts appear wrinkled (like crumpled cellophane). Oocysts in stool are unsporulated and do not contain any internal definition or structure. Resemble oocysts of cryptosporidium except that the latter are smaller and have definite internal structures.

***Microsporidium spp.* – oocysts** [39,19]

Size: varies according to the species.

- *Encephalitozoon, Vittaforma corneae, Nosema spp.* – 1.5-4 µm
- *Enterocytozoon bieneusi* – 0.8-1.4 µm

Although *Microsporidia* are acid-fast, their small size makes their recognition very difficult in modified acid-fast stained smears. In modified Trichrome stained smears, oocysts of *Microsporidia* appear small, oval refractive spores with bright pinkish red walls. Often, a belt-like stripe, which also stains pinkish red, is seen in the middle of the stripe.

Agar Plate Culture Method for Strongyloides stercoralis [39]

Principle:

As the larvae crawl away from the central stool inoculum on to the surface of the agar, they carry bacteria with them and leave them on their paths on agar surface. Bacteria grow on the agar to form colonies which appear as tracks.Live, motile larvae can also be seen. Final confirmation of larval identification is made via wet examination of the sediment from the

formalin washings.

Procedure:

- Approximately 2 g of fresh stool was placed in the center of the nutrient agar plate (area approximately 1 inch in diameter). The plate was closed with lid and was sealed with cellulose tape.
- The agar plate was kept at room temperature for 2 days (with right side up). After 2 days, the sealed plate was examined through the lid under the microscope for microscopic colonies that develop as random tracks on the agar and evidence of larvae at the ends of the tracks away from the stool. Plate was examined daily for at least 6 consecutive days, if no positive findings are found.
- With the ends of hot forceps, a hole was made in the top of the plastic petri dish. 10 ml of 10% formalin was gently added through the hole on the agar and swirled to cover the whole surface and rinse. Allowed to stand for 30 min.
- Plate was opened and formalin rinse fluid was transferred to a centrifuge tube and centrifuged at 500Xg for 5 min.
- A wet mount was prepared with the sediment and was examined under microscope for *Strongyloides stercoralis* larvae in low and high dry objectives.

Preservation of Feces specimens [40,39]

All the samples were preserved using 10% formaldehyde solution. Three parts of 10% formaldehyde solution was added to one part of the specimen and mixed well by crushing the specimen with a glass rod. Lid of the container was closed air tight and stored at room temperature.

All the specimens, either fresh or preserved, irrespective of the observations in the above procedures, were subjected to **Concentration Technique** as follows.

Formalin Ether sedimentation concentration Technique [39]

- A half teaspoon (about 4 g) of fresh stool was transferred into 10 ml of 5 to 10% formalin in a wide mouthed container.
- The stool and formalin were mixed thoroughly and the mixture was allowed to stand for a minimum of 30 min for fixation. If the specimen was already in 5 or 10% formalin, the stool preservative mixture was stirred and used for the next steps.
- Depending on the amount and viscidity of the specimen, a sufficient quantity was strained through wet gauge (No more than 2 layers) into a conical 15-ml centrifuge tube to give the desired amount of sediment (0.5 to 1 ml). Usually, 8 ml of stool formalin mixture is sufficient.
- 0.85% NaCl was added (or 5 or 10% formalin) almost to the top of the tube, and centrifuged for 10 min at 500 × g. The amount of sediment obtained should be approximately 0.5 to 1 ml.

- The supernatant fluid was decanted and discarded. The sediment was resuspend in saline or formalin and saline (or formalin) was added almost to the top of the tube, and centrifuged again for 10 min at 500 × g. This second wash may be eliminated if the supernatant after the first wash is clear or light tan.
- The supernatant fluid was decanted and discarded, and the sediment at the bottom of the tube was resuspended in 5 or 10% formalin. The tube was filled half full only.
- Then 4 to 5 ml of ethyl acetate was added. The tube was stoppered and was held so that the stopper is directed away from face and shaken vigorously for at least 30 s. After a 15- to 30- s wait, the stopper was carefully removed.
- It was centrifuged for 10 min at 500 × g.
- Four layers resulted:
    i. a small amount of sediment (containing the parasites) in the bottom of the tube
    ii. a layer of formalin
    iii. a plug of fecal debris on top of the formalin layer and
    iv. a layer of ethyl acetate at the top.
- The plug of debris was freed by ringing the plug with an applicator stick; decanted and discarded all of the supernatant fluid. After proper decanting, a drop of or two of the fluid remaining on the side of the

tube may run down into the sediment. This fluid was mixed with the sediment. If the sediment was still somewhat solid, 1 or two drops of saline (or formalin) was added to the sediment, mixed.

This concentrated material was used for preparing saline wet mount, Iodine wet mount and lactophenol cotton blue wet mount and examined for the parasites. Smears were prepared from the concentrated material and stained using modified Kinyoun's acid-fast staining method and modified trichrome method and examined for parasites.

Diagnosis of HIV infection

Sample collection

Serum samples were used for detecting antibodies to HIV virus antigens. After pretest counseling and informed written consent was obtained, with all aseptic precautions 3-5 ml of venous blood was collected and transferred into a properly labeled sterile plastic leak proof specimen container with screw cap. The blood was allowed to clot for 30 min at room temperature. The vial was centrifuged at 3000 rpm for 10 min to separate serum to avoid hemolyis. The serum was transferred to a sterile plastic screw capped leak proof tube [22,23].

Appropriate NACO strategy was used to determine HIV status of the individuals. Initially highly sensitive screening test was used. The sample was considered negative if the test gave non-reactive result. In case the test result was reactive the same sample was tested with another test kit (based on

different principle of test or having different antigens compared to the first test). If the result was reactive with second test kit also the ample is considered to be positive. In case the sample was positive by first test kit ant negative by second test kit, sample was subjected to a tiebreaker third test. If third test is reactive, sample was reported as indeterminate and follow-up testing was undertaken after 2-4 weeks. In case the tiebreaker third test was negative, sample was reported as negative [22,23].

MICROLISA – HIV

Microwell ELISA Test for the detection of Antibodies to HIV-1 and HIV-2 in Human Serum/Plasma.

Principle

Microlisa HIV test is an enzyme immunoassay based on indirect ELISA. HIV envelope proteins gp41, C terminus of gp 120 for HIV-1 and gp 36 for HIV-2 representing immunodominant epitopesare coeated on to microtiter wells. Specimens and controls are added to the microtiter wells and incubated. Antibodies to HIV-1 and HIV-2 if present in the specimen, will bind to the specific antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated antihuman IgG is added to each well. This conjugate will bind to HIV antigen-antibody complex present. Finally substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of HIV-1 and/or HIV-2

antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader before absorbance at a wavelength of 450 nm. If the sample does not contain HIV-1 or HIV-2 antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

Test Procedure

- 100 µl sample diluent was added to well A-1 as blank.100 µl negative control was added to well no. B-1 and C-1 each. 100 µl positive control was added to D-1, E-1 and F-1 wells.
- 100 µl sample diluent was added to each well starting from G-1 followed by addition of 10 µl sample. Cover seal was applied and incubated at 37ºC for 30 min.
- After incubation time was over, wells were washed with working wash solution for 5 times (300 µl, 30 sec soak time).
- 100 µl working of working conjugate solution is added in each well including the blank. Cover seal was applied and incubated at 37ºC for 30 min. Then it was washed 5 times as before.
- 100 µl of working substrate solution is added in each well including A-1. Incubated at room temperature for 30 min, in dark.
- 50 µl of stop solution was added to all the wells and absorbance was read at 450 nm within 30 min.

Test Validity

1. Blank must be < 0.100 2. Negative Control mean must be ≤ 0.150

3. Positive Control mean must be ≥ 0.50

Calculation of Results

Cut Off value = (Mean of Neg. Control + Mean of Pos. Control)/ 6

Interpretation of Results

Test specimens with absorbance value less than the cut off value were considered non-reactive and were considered negative for anti-HIV antibodies. Test specimen with absorbance values greater than or equal to cut off value were considered reactive for anti-HIV antibodies.

Performance Characteristics of Test Kit

Sensitivity 100% Specificity 99.5%

HIV EIA COMB

Rapid visual EIA test for the Qualitative and Differential Detection of Antibodies to HIV-1 (including subgroup O and subgroup C) and HIV-2 in Human Serum/Plasma.

Principle

HIV antigens are immobilized circular spot on the polystyrene comb. When incubated with a specimen containing HIV-1 and/or HIV-2 antibodies, these antibodies bind specifically to the immobilized antigens. The comb is washed to remove unbound antibodies. The comb is then placed in microwells

containing enzyme conjugate (alkaline phosphatase conjugated anti-human IgG). This conjugate will bind to antigen antibody complex present on the comb. Finally the comb is placed in microwells substrate and is incubated. The bound conjugate will react with substrate. The results are directly visualized by the presence of distinct grey-blue dot(s) on the surface of comb.

Setting up of the test

- 250 µl of sample diluent was added to required number of microcuvettes using the dropper provided with the kit. One drop (50 µl) of sample (serum) was added to respective above microcuvettes. The sample was mixed with the diluent by repeatedly aspirating and expelling with dropper. The position and identity of the samples was recorded as they were added.
- Another required number of new microcuvettes in another row were filled with 300 µl of wash buffer.
- Another required number of new microcuvettes in another row were filled with Enzyme conjugate directly from the vial.
- Again another required number of new microcuvettes in another row were filled with 300 µl of wash buffer.

Test Procedure

- Required number of combs were taken and sample numbers marked.
- The comb was placed into the microcuvettes containing samples and incubated for 8 min at room temperature. During incubation comb was withdrawn and inserted in the microcuvettes for 15 sec.
- The comb was removed and tips were blotted on absorbent material.
- The comb was placed into microcuvettes containing wash buffer washed for one minute by carefully mixing the comb up and down in the wash solution. The comb was removed and the tips blotted.
- The comb was placed into microcuvettes containing Enzyme conjugate and incubated for 8 min at room temperature. During incubation comb was withdrawn and inserted in the microcuvettes for 15 sec. The comb was washed in wash buffer as before and blotted.
- Then it was placed into microcuvettes containing chromogenic substrate and incubated for 8 min at room temperature. The comb was removed and the tips blotted. Again the comb was washed as before and the tips blotted.
- The comb was placed into microcuvettes containing Substrate and incubated for 8 min at room temperature. During incubation comb was withdrawn and inserted in the microcuvettes for 15 sec.
- The comb was removed and the tips blotted. Again the comb was washed as before and the tips blotted.

- The comb was washed in distilled water and the tips blotted.
- The comb was placed on a clean surface, reactive side up and allowed to air dry. Results were read only after the comb was completely dry.

Validity - Appearance of distinct grey-blue dot in the control position indicates the test is valid.

Interpretation of Results

Non-reactive - If only one dot (in the control position) appears, the sample is non-reactive.

Reactive - If two dots, one for the control and the other for HIV1appeared, the sample was considered to be reactive for antibodies to HIV-1.If two dots, one for the control and the other for the HIV-2 appeared, the sample was considered to be reactive for antibodies to HIV-2.If all the three dots, one each for control, HIV-1 and HIV-2 appeared**,** the sample was considered to be reactive for antibodies to both HIV-1 and HIV-2.

Performance Characters: Sensitivity – 100%; Specificity – 99.9%

DIAGNOS HIV BI-DOT TEST

Rapid visual test for the Qualitative detection of antibodies to HIV-1 and HIV-2 in human serum/plasma. (J.Mitra & Co.Pvt.Ltd., New Delhi, India)

Principle: HIV antigens (gp41, C terminal of gp 120 & gp 36 representing the immunodominant regions of HIV-1 and HIV-2 envelope gene structure respectively) are immobilized on a porous immunofiltration membrane and are absorbed into the underlying absorbent. As the patient sample passes

through the membrane, HIV antibodies, if present, bind to the immobilized antigens. Conjugate binds to the Fc portion of the HIV antibodies to give distinct pinkish purple dot against a white background.

Test Procedure:

- 3 drops of Buffer Solution are added to the centre of the device.
- The sample dropper provided with the kit is held vertically and 1 drop of patient's sample (serum/plasma) is added. 5 drops of Buffer Solution are added.
- 2 drops of Liquid Conjugate are added directly from the conjugate vial.5 drops of Buffer Solution are added and results are read immediately.

Interpretation of the Results

Non-reactive – If only one dot (the Control Dot) appears, the specimen I non-reactive for antibodies either to HIV-1 or HIV-2.

Reactive – If 2 dots, one for control and the other for test appear, the specimen is reactive for antibodies to HIV-1 and/or HIV-2.

Performance Characteristics: Sensitivity – 100% ; Specificity – 100%

ESTIMATION OF CD_4 COUNT [52,36]

Sample -CD_4 count was estimated form 5 ml of K_2EDTA blood collected from each patient and was processed the same day. The CD_4 count was done by automated flow cytometry analyzer FACS calibur (Beckton Dickinson).

Principle of Flow Cytometry

Signals are generated by cells or particles in suspension passing through a light (usually LASER) source in the flow cell, in a single file (aligned by Sheath Fluidic System) and are analyzed electronically in a flow cytometer. The parameters measurable include forward scatter (an indicator of cell size), side scatter (an indicator of granularity of the cell) and signals from multiple fluorescent dyes (e.g. FITC, phycoerythrin) tagged to cell surface phenotypic marker-specific antibodies.

BD FACSCalibur is a flow cytometer which is capable of measuring the scatter and the fluorescence parameter. It can detect the scatter parameter namely the forward and the side scatter which gives information about the size and granularity of the cell. The BD FACSCalibur can detect up to 3 fluorescence parameters. It can measure both absolute CD4 + T-lymphocyte count as well as % CD4 count.

Antibody panels - BD TriTEST CD3 fluorescein isothiocyanate (FITC)/CD4 phycoerythrin (PE)/CD45 peridinin chlorophyll protein (PerCP) wasused, which is a three-color direct immunofluorescence reagent to identify and determine the percentages and absolute counts of mature human T lymphocytes (CD3) and helper/inducer (CD3+CD4+) T-lymphocyte subsets in erythrocyte-lysed whole blood. When used with TruCOUNT Tubes, absolute counts of these populations can be enumerated from a single tube.'

Procedure

- Required numbers of BD Trucount Tubes were taken. 20 µl of Tritest antibody reagent is added to each tube, by placing it on the side wall of the tube, to avoid disturbing the bead at the bottom.
- 50 µl of well mixed whole blood collected in K_2EDTA is added to each tube in a similar way and vortexed.
- Incubated in dark at room temperature for 15 minutes.
- 450 µl of 1X Lysing solution was added and vortexed.This cause lysis of RBCs and fixation of cells on the beads.
- Incubated in dark at room temperature for 15 minutes.
- Reading was obtained on the BD FACSCalibur.

Statistical Analysis

A comparison of frequency of parasites between cases and controls and also among cases with different diarrhoeal status was performed by chi-square test. The significance of association of CD4 counts and duaration of diarrhea was analysed by one-way ANOVA test. P value of <0.05 was considered statistically significant.

RESULTS

A total of 150 individuals were enrolled in this study, conducted during October 2007 to November 2008 at Dept. of Microbiology, Govt. Stanley Medical College, Chennai. It included 100 HIV seropositive cases and 50 individuals without clinical manifestations of HIV/AIDS as controls. At least two stool samples were collected from all the subjects and were examined for coproparasites by saline, iodine and lacto-phenol cotton blue (LPCB) wet mounts and permanent staining methods like modified Kinyoun's acid-fast staining and modified Trichrome staining. Parasites were observed in samples of 33 cases and 8 control subjects.

Results were analyzed further as follows:

Study Population:

TABLE – 1
Description of study groups

Study group	Description	Number of individuals
Cases	HIV/AIDS patients with or without diarrhoea	100
Controls	Clinically Non-HIV/AIDS	50

Cases included 100 randomly selected HIV seropositive patients with and without diarrhoea and other gastrointestinal symptoms. Control group had 50 subjects not having clinical symptoms and signs of HIV/AIDS.

TABLE -2
Age and Sex Distribution of Study Population

Age (Years)	HIV positive		Control group	
	Male (%)	Female (%)	Male (%)	Female (%)
<20	2(3.26)	1(2.63)	5(17.86)	3(13.64)
20-30	7(11.29)	10(26.32)	9(32.14)	8(36.36)
30-40	34(54.84)	18(47.37)	11(39.29)	7(31.82)
40-50	14(22.58)	7(18.42)	2(7.14)	2(9.09)
>50	5(8.06)	2(5.26)	1(3.57)	2(9.09)
Total	62	38	28	22

Both the study groups included individuals of all age groups and sexes. Cases included 62 males and 38 females and control group had 28 males and 22 females. (Cases included two eunuchs and for the convenience of analysis they were considered as females). Among the cases, maximum subjects were found to be in the age group of 30-40 years, with 34 males and 18 females. In the control group also, maximum subjects were in 30-40 years age group with 11 males and 7 females.

In HIV infected group, majority of the men were labourers (40.32%) followed by drivers (19.35%) and farmers (17.74%). Housewifes (52.63%) constituted the major portion of HIV infected, followed by farmers accounting for 31.58%.

TABLE - 3
Distribution of Diarrhoea in HIV infected individuals

Gender	Acute Diarrhoea	Chronic Diarrhoea	Without Diarrhoea
Male	25	20	17
Female	13	10	15
Total	38	30	32

The HIV positive individuals included 25 males and 13 females with diarrhoea lasting less than 2weeks (Acute Diarrhoea) and 20 males and 10 females had diarrhoea for more than 2 weeks (Chronic diarrhoea).Remaining cases were free from diarrhoeal symptoms(17 males and 15 females).

Distribution of Parasites in the Study Groups:

27.33% of the study population showed one or more enteric parasites. Among the 100 cases examined for coproparasites 33 (33%) showed parasites and 8 (16%) out 50 controls had coproparasites.

TABLE - 4
Prevalence of intestinal parasites

Study group	Total	Positive	Percentage
Cases	100	33	33
Controls	50	8	16
Total	150	43	28.67

Infection rate was higher in HIV positive individuals compared to the control group and it was statistically significant (Chi-square test, p=0.037).

TABLE -5
Distribution of Parasites in the Study Population

Parasites	Cases (n=100)		Controls (n=50)	
	No. of Parasites	**%**	**No. of parasites**	**%**
Protozoa	**32**	**32**	**2**	**4**
E. histolytica/dispar	5	5	1	2
E.coli	2	2	1	2
*G.*intestinalis	1	1	0	0
Isospora belli	21	21	0	0
Cryptosporidium spp.	2	2	0	0
Microsporidium	1	1	0	0
Helminths	**4**	**4**	**6**	**12**
Ascaris lumbicoides	0	0	3	6
Hook worm	2	2	1	2
Trichuris trichura	0	0	2	4
Strongyloides stercoralis	2	2	0	0

In HIV positive individuals, out of 36 parasites seen, 32(32%) were protozoa and 4 (4%) were helminths, accounting for 88.89% and 11.43% of total parasites detected respectively. Protozoan infections were significantly more common in cases than in controls ($p<0.001$, Chi-square test). *Isospora belli* was the commonest parasite found among cases, comprising 58.33% (21) of all the parasites detected (21% cases), followed by *Entamoeba histolytica/dispar* 13.89% (5) and *Entamoeba coli*, *Cryptosporidium spp*., *Strongyloides stercoralis* and hook worm which comprised 5.56% each of all parasites detected in HIV infected individuals. *Microsporidia and Giardia lamblia* cysts were detected in one patient (2.78%) each. Statistically significant difference in rate of infection among

cases and controls was found only for *Isospora* ($p<0.001$, Chi-square test).

Among the control group studied, helminths were detected in 6 (75%) subjects and protozoa in 2 (25%) subjects. *Ascaris* was the commonest parasite in control group comprising 37.50% (3), followed by *Trichuris trichura* 25% and hook worms 12.50% of all parasites in control group. Protozoa, E.*histolytica*/*dispar* and E.*coli* were seen in one case each (12.50%). Helminthic infections were significantly more common in controls than in cases ($p=0.046$, Chi-square test).

Enteric parasites were found in higher numbers in males (40.32%) than in females (18.40%), among HIV positive individuals. The infection rate was 14.29% and 18.20% in males and females in the control group. Highest infection was observed in 30-40 years age group among HIV infected individuals, but it was not statistically significant when compared to other age groups ($p=0.866$, Chi-square test).

TABLE -6
Distribution of Parasites among Different Age groups and Gender in Study Population

Age (years)	Cases (n=100)						Controls (n=50)					
	Male			Female			Male			Female		
	Total	Positive	%	Total	Positive	%	Total	Positive	%	Total	Positive	%
<20	2	0	0	1	0	0	5	1	3.57	3	1	4.55
20-30	7	4	6.45	10	1	2.63	9	1	3.57	8	3	13.6
30-40	34	16	25.81	18	5	13.16	11	1	3.57	7	0	0
40-50	14	2	3.23	7	1	2.63	2	1	3.57	2	0	0
>50	5	2	3.23	2	1	2.63	1	0	0	2	0	0
Total	62	25	40.32	38	7	18.4	28	4	14.29	22	4	18.2

Intestinal parasitic infection was more common in those with diarrhoea (44.12%) than those without diarrhoea (15.62%) in HIV infected individuals. Among HIV seropositive subjects with diarrhoea, those having chronic diarrhoea showed more parasitic infections (60%) compared to those with acute diarrhoea (31.58%).

TABLE – 7
Association of total Parasites and Diarrhoea in HIV infected individuals

Diarrhoea	Total	Positive	%
Without Diarrhoea	32	5	15.62
Acute Diarrhoea	38	12	31.58
Chronic Diarrhoea	30	19	63.33

The difference in infection rates among different diarrhoeal groups was significant (Chi-square test, p=0.027).

TABLE – 8
Distribution of various parasites in relation to diarrhoea

Parasites	Acute diarrhea (n=38)		Chronic diarrhea (n=30)		Without diarrhea (n=32)	
	No. of parasites	%	No. of parasites	%	No. of parasites	%
Protozoans						
E. *histolytica*	1	2.63	3	10	1	3.13
E.*coli*	0	0	1	3.33	1	3.13
Giardia lamblia	0	0	1	3.33	0	0
Isospora belli	6	15.79	12	40	3	9.38
Cryptosporidium spp.	2	5.26	0	0	0	0
Microsporidium spp.	0	0	1	3.33	0	0
Helminths						
Ascaris lumbricoides	0	0	0	0	0	0
Hook worm	1	2.63	1	3.33	0	0
Trichuris trichura	0	0	0	0	0	0
Strongyloides stercoralis	2	5.26	0	0	0	0
Total	12	31.58	19	63.33	5	15.63

Isospora belli was found in those with and without diarrhea. It was significantly more common among those with chronic diarrhoea (40%, p<

0.001, Chi-square test) than those with acute diarrhea (15.79%) and it was also found among 9.38% of HIV patients without diarrhoea. *Cryptosporidium spp*. and *Strongyloides stercoralis* were seen in 5.26% each of cases with acute diarrhoea only. *Microsporidium* was seen in one case with chronic diarrhea. All these opportunistic parasites were not detected in the control group.

TABLE -9
Types of Parasitic infections in study population

Results	Cases		Control		Total	
	No. of parasites	%	No. of parasites	%	No. of parasites	%
Pathogenic	33	94.29	7	87.5	40	93.02
Opportunistic	25	71.43	0	0	25	58.14
Nonpathogenic	2	5.71	1	12.5	3	6.98

(Pathogenic group included both opportunistic and non-opportunistic potential pathogenic parasites)

Majority (93.18%) of the parasites identified in the stool samples of both cases and control groups were pathogenic (94.44% and 87.5% in cases and controls respectively). Opportunistic intestinal parasites contributed to statistically significant portion (71.22%, $p< 0.001$, Chi-square test) of the parasites detected among the HIV infected individuals,.

TABLE- 10
Single and Dual parasitic infections in Study Subjects

No of parasites	Cases (n=100)	%	Controls (n=50)	%
Single	30	30	8	16
Dual	3	3	0	0

Among all subjects positive for coproparasites, 30 of cases and all among the control group had single parasitic infection. Dual infection was observed in 3 HIV infected persons and no dual infection was seen in control group.

Association with the Immune Status:

TABLE -11
Immune status and Diarrhoea in HIV positive individuals

Diarrhoea	Average CD4 count (cells/mm^3)
Acute Diarrhoea	239.26
Chronic Diarrhoea	179.1
Without Diarrhoea	319.93

One-way ANOVA, p= 0.011

Mean CD4 counts among HIV infected and not having diarrhoea were significantly higher (319.93, p=0.011,) compared to those with diarrhea in the same group. Among HIV infected with diarrhoeal symptoms, those with chronic diarrhoea had significantly lower CD4 counts (179.10cells/mm^3, p=0.011) compared to those with acute diarrhoea (239.26 cells/mm^3).These difference were statistically significant (One-

way ANOVA, p= 0.011).

TABLE -12
Opportunistic Parasites in HIV positive individuals and CD4 counts

CD4 counts (cells/mm^3)	**<100**		**100-200**		**200-500**		**> 500**	
Parasites	Diarrhoea	No Diarrhoea	Diarrhoea	No Diarrhoea	Diarrhoea	No Diarrhoea	Diarrhoea	No Diarrhoea
Isospora belli	2	0	8	1	5	0	3	2
Cryptosporidium spp.	2	0	0	0	0	0	0	0
Strongyloides stercoralis	2	0	0	0	0	0	0	0
Microsporidium spp.	1	0	0	0	0	0	0	0
Total	6	0	8	1	5	0	3	2

Isospora belli was detected in subjects with wide range of CD4 counts, but the rate was significantly higher in those with CD4 counts less than 200 cells/mm^3 (p=0.042, Chi-square test). *Cryptosporidium spp*., *Microsporidium spp*. and *Strongyloides stercoralis* infections were seen in those with CD4+ counts less than 100 cells/mm^3.

TABLE - 13
Parasites identified by different methods

Of different methods employed in the study to detect coproparasites, saline wet mount, iodine wet mount, LPCB wet mount were able to detect 30 parasites and mod. Acid-fast stain could detect 24 parasites.Mod. Trichrome stain could identify one parasite. Out of 21 *Isospora belli* detected, all were seen in mod. Acid-fast staining and 10 were visualized in wet mounts (P=0.023).

TABLE - 14
Detection of coproparasites before and after Formalin Ether Sedimentation Concentration procedure

BC=before concentration, AC=after concentration

Detection of parasites in stool samples after Formalin Ether Concentration procedure was improved in all the methods employed in both the cases and the control group. Saline wet mount could detect 16 and 22 parasites before and after the concentration procedure. Similarly, iodine wet mount detected 19 & 22, LPCB wet mount detected 17 & 22 and mod. Acid-fast staining method could detect 20 & 23 parasites, before and after concentration procedure respectively.

DISCUSSION

Diarrhoea is a common complication of HIV positive patients inducing weight loss and cachexia and occurs in 90% of AIDS patients in developing countries. Enterocyte / neural dysfunction related to HIV infection may be responsible for diarrhoea in some. Further, progressive decline in their immunological responses makes them extremely susceptible to a variety of common and opportunistic infection.[35]

The clinical course and pattern of opportunistic infections (OI) varies from patient to patient in different areas of India. Opportunistic intestinal parasitic infections should be suspected in any HIV-infected patient with advanced disease presenting with diarrhoea. Intestinal parasites such as *Cryptosporidium parvum*, *Isospora belli* lead to increase in morbidity and mortality in HIV/AIDS cases. Also, CD4 cell counts have been significantly lower in these individuals with diarrhoea than in those without.[12]

In recent years, numerous studies have outlined the emergence of important gastrointestinal protozoa like *Cryptosporidium parvum*, *Isospora belli*, *Cyclospora*, *Microsporidium* and also helminths as causative agents of diarrhoea in HIV/AIDS.[35] Some studies also have shown isolation of *Cryptosporidium* and *Cyclospora* in immunocompetent individuals. [48]

1. Distribution of diarrhoea in the study groups

In the present study, 68% of HIV positive individuals had diarrhoea,

which included 38 males and 30 females. Those having diarrhoea for less than 2 weeks were considered to have acute diarrhoea and diarrhoea lasting more than 2 weeks was considered to be chronic.[15] Similar definitions of diarrhoea were followed in studies conducted by Mukhopadhya *et al* [48] and Dwiwedi KK *et al* [15]. 25 of males and 13 females among HIV infected with diarrhoea had acute form of it and the remaining were suffering from chronic diarrhoea; acute diarrhoeac subjects outnumbered chronic diarrhoea.

3. Distribution of parasites in the study groups

The overall infection rate in this study was 27.33%. 33% of cases and 16% of controls showed enteric parasites and the difference was significant.

The infection rates observed in the present study were comparable to the studies of Ballal M [3], Ramakrishnan *et al* [57], Prasad KN *et al*. [56], Vignesh R *et al*.[66],Botero JH *et al*.[6]and Sang-Mee Guk *et al*.[60]

Mukhopadhya *et al*. (58.56%)[48], Dwiwedi KK *et al*. (62.7%)[15] and Hailemariam *et al*.(52.6)[26] have reported higher infection rates in HIV positive patients. Figures from various studies have demonstrated striking geographic variations in the prevalence of enteric parasites and also variations with time in the same geographical area [48].

TABLE 15
Infection rates of Enteric Parasites in different studies

In the present study, Parasites were found in higher numbers in males (40.32%) than in females (18.40%), among HIV positive individuals. The infection rate was 14.29% and 18.20% in males and females in the control group. Male preponderance in infection rates among HIV infected has been shown in studies by Dwiwedi K *et al*[15]. and Mohandas *et al*[46]. with 76% and 64% males infected respectively.

In this study, higher parasitic infection rate was seen in 30-40 years age group in HIV positive subjects, with 25.81% male and 10.50% females infected being in that age group. In the study by Mukhopadhya *et al*[48] mean age of infected individuals was found to be in 30-40 years age group[48].Ramakrishnan K *et al*. and Aggarwal *et al*. have reported infection rate of intestinal parasites in HIV patients to be higher in reproductive age group (16-45). [57,1]

4. Types of parasitic infections

Majority of the enteric parasites identified in both cases and control groups were pathogenic (94.44% and 93.18% respectively).Opportunistic intestinal parasites contributed to significant proportion of the parasites detected among the HIV infected individuals (72.22%).

In the present study 30 (90.90%) of the cases and all the controls were found to be infected with single intestinal parasite. Among the HIV infected, 3

(9.09%) were having infection by 2 parasites and no dual infection was found in control group. This observation is comparable to the observations in a south Indian study by Vignesh R *et al*, who reported multiple infections in 2% cases.[66] Studies conducted by Mukhopadhya *et al*.(21% in diarrheoa and 8% in non-diarrhoeal cases), Ramakrishnan K *et al*. (HIV 22.5% and Non-HIV 12.5%) and Hailemariam G *et al*. (HIV 36.59% and Non-HIV 18.8%) reported higher prevalence of multiple infections [48,57,26].

In the present study, protozoa accounted for 31% and helminths 4% in cases. Among controls, helminths (12%) were commoner than protozoa (4%). Many previous studies also have shown that protozoal infections are commoner than helminthic in HIV patients.[48,35]

5. Spectrum of parasites identified in the study population

In this study, Isospora belli was the commonest (21%) enteric parasite identified in HIV patients followed by *E.histolytica* (5%), *Cryptosporidium spp.* (2%), *S. stercoralis* (2%), *E. coli* (2%), *Microsporidium spp.* and *G. lamblia* (1%) respectively.

In the control group, most common parasite identified was *A. lumbricoides* (6%) followed in frequency by *T. trichura* (4%), Hook worm (2%), *E. histolytica* (1%) and *E.coli*(1%).

Higher prevalence of Isospora compared to other parasites, among south Indian HIV infected patients has been documented by studies conducted by Kumar SS *et al*. (14%), Vignesh R *et al*. (26.1%) and Mukhopadhya *et*

al.[35,66,48] Our observations match with those of Vignesh R *et al*.

Studies conducted in the other parts of India have shown *Cryptosporidium* spp. as the predominant coccidian parasite in the HIV-infected and prevalence of Isospora belli is found to be lesser, as in observations by Ballal M (18.57%, 2.86%), Aggarwal A *et al* (13.63%,6.06%), Talib SH *et al* (6.25%,0%), and Dwiwedi K *et al* (33%,2.7%).[3,1,15,63] In the present study *Cryptosporidium spp*. was found in 2% of HIV infected and was comparable to study of Vignesh R *et al* (2.9%) conducted in Chennai.

Vignesh R *et al* have found a statistically significant increase in cases of Isospora belli and a decline in number of Cryptosporidial cases in HIV patients in Chennai during 2003-2006. Changes in the trend with time was hypothesized to be due to changes in residence, patient population and weather conditions (rainfall and temperature).[66]

Opportunistic parasites *Isospora belli, Cryptosporidium* and *Strongyloides* were found only in HIV infected patients in the present study and conventional parasites were found in both the groups. Lindo JF *et al* also found *Cryptosporidium* and Strongyloides stercoralis only in HIV infected patients.[38] Infection by opportunistic parasites have been documented in non-immunocompromised subjects also, especially *Cryptosporidium* pp., but that is less common.[57,3] Another coccidian parasite, Cyclospora Cayetanensis was not found in the present study, its low incidence among HIV patients in south

India has been documented .[35, 48]

Certain parasites are found more commonly in HIV-infected patients than the other. The reasons for these patterns are not clear. It has been postulated that colonization of intestinal tract by parasites may be influenced by enteropathy induced by infection with HIV. Virus-induced structural and functional impairment leading to common gastrointestinal symptoms has been shown to occur independently of infection with enteric pathogens. This may selectively deter the establishment and or survival of extracellular and luminal parasites. In contrast, where as gut of HIV-infected individuals may not be a favorable environment for the establishment and/or survival of extracellular parasites, intracellular and mucosal dwelling organisms may not be adversely affected by the pathologic changes. The frequency of infection with *C. parvum*, *S. stercoralis* and I. belli has been associated with increasing duodenal mucosa damage.[38]

The lower prevalence of extracellular helminths infections in HIV-infected individuals, as compared with those who are HIV negative, may actually be due to loss of infection. Both progression of HIV infection to AIDS and helminths infections are associated with increased T-helper cell 2 (Th2) cytokine production. The preponderance of these factors as AIDS progresses may contribute to decreased worm survival.[38]

Apart from *I. belli, Entamoeba histolytica/dispar* cysts were seen in an HIV patient without diarrhoea, suggesting possibility of it being non-

pathogenic *E. dispar*, but no further evaluation was done to confirm it.

Figures from various studies demonstrate striking geographic variations in the prevalence of individual pathogens in HIV patients. These variations may relate both to the prevalence of pathogens within the community, and to drugs used prophylactically in patients with HIV infection. Moreover, the quantitative differences in parasites infecting HIV patients with diarrhoea may be due to differences in genotypic / phenotypic characters.

6. Diarrhoea and intestinal parasitic infection

In our study, higher parasitic infection was found in those with diarrhoea (44.12%) than those without diarrhoea (15.62%) among HIV infected individuals. Similar observations were made in studies conducted by Kumar SS *et al*. (39% and 14%), Mukhopadhya *et al*. (57.4% and 40%) and Botero JH *et al*.(32.4% and 4%) [35,48,6].

Those having chronic diarrhoea showed significantly higher parasitic infections (63.33%) compared to those with acute diarrhoea (31.58%) in the current study. Ramakrishnan *et al*. found enteric parasites in 38.7% of HIV patients with chronic diarrhoea.[57] Dwiwedi *et al*. showed higher infection rates among those with chronic diarrhoea (88.9%) than in acute diarrhoea (52.2%) [15].

In the current study, *I. belli* was significantly more common in chronic diarrhoea (40%) than in the acute (15.9%) and it was also seen in 9.38% asymptomatic HIV-infected persons. Kumar *et al* have documented higher

prevalence of *I. belli* in chronic diarrhoea (18.6%) than in acute diarrhoea (7.3%) in HIV infected patients, but prevalence in each group was lower than that in our study [35].

C. parvum and *S. stercoralis* were found in acute diarrhoea cases only (5.26% each) and *Microsporidium spp.* was seen in a chronic diarrhoea case. Previous studies have shown Strongyloides to be less common in general population (0.4%) as well as in HIV-infected individuals (0.9-6.3%) in India except in Manipur (27.3%). [15]

7. Diarrhoea and immune status of the HIV positive patients

Among cases, mean CD4 counts were found to be significantly lower in those with diarrhoea (209.18%) than those without (319.93 cells/mm^3) in this study. Those with chronic diarrhoea (179.1cells/mm^3) had significantly lower CD4 counts compared to acute diarrhoea (239.26 cells/mm^3).

In the study conducted by Zali MR *et al*., they found mean CD4 counts to be lower in those with diarrhoea (327.17±174.44 cells/mm^3) than those without (523.14±239.12 cells/mm^3) but the mean CD4 counts in each group were lower than those in our study.[70] Similar observations were made by Ramakrishnan K *et al.* [57]

Mean CD4 counts were observed to be 141 cells/mm^3 in those with diarrheoa (265 cells/mm^3 in acute and 123 cells/mm^3 in chronic) and 390 cells/mm^3 in those without diarrhoea in the study of Dwiwedi KK *et al.* [15]

8. Opportunistic parasites and immune status of cases

Maximum number of opportunistic parasites was seen in those with CD4 counts less than 200 cells/mm^3.The prevalence of parasites was lesser in CD4 counts of 200-500 and >500 cells/mm^3.Of 100 cases 2 *Strongyloides* were identified in patients with CD4 <100 cells/mm^3 and 2 *Cryptosporidium* spp. were seen in patients with CD4 <50 cells/mm^3. I. belli was found at all CD4 ranges and 42.86% of total *I. belli* detected were from cases with CD4 counts 100-200 cells/mm^3.

Studies by Wiwanitkit V, showed highest prevalence of opportunistic intestinal parasite infection in patients with a low immune level CD4 < 200 / mm^3 and with diarrhea.[69] Meamar *et al*. have reported mean CD4 counts to be 137±23.8 cells/mm^3 for *I. belli* and 50.1±8.8 cells/mm^3 for *Cryptosporidium* spp.[42] Dwiwedi KK *et al* have reported that the extent of deterioration of immunity, as measured by CD4 count could predict the presence of coccidian parasites with or without multiple infections[15]. In a study by San-Mee Guk *et al*, CD4 counts associated with Cryptosporidial infections were 50 cells/mm^3 or less and those for I. belli were 131 cells/mm^3 or higher.[60] Botero JH *et al*. have observed that patients who presented with Microsporidia have CD4 T lymphocytes of less than 50 cells/mm^3. [6]

9. Methods for detection of enteric parasites

At least 2 freshly passed stool specimen were collected without preservative and processed for enteric parasites. Cartwright CP, in his study on 1,374 subjects concludes that in populations with a high prevalence of intestinal parasitic infections, 2 independently collected stool specimens should be subjected to ova and parasitic examination to ensure adequate diagnostic sensitivity. Most parasitology textbooks and manuals recommend the examination of at least 3 independently collected stool specimens to maximize the sensitivity and such recommendations are based on older studies[9].

In this study, saline, iodine and LPCB wet mounts were used for ova and cyst identification. Modified Kinyoun's acid-fast (MKAF) staining was done for coccidian parasites and Modified Trichrome staining was done for *Microsporidium spp*.

Sensitivity of all the three wet mounts was similar in detection of conventional parasites and *Isospora*. Cysts were better visualized in iodine and LPCB mounts compared to saline mounts. In general, structures were more discernible with iodine and LPCB mounts compared to saline mounts. While saline and iodine mounts got dried fast, LPCB mounts could be examined even after a few hours.

Motile larvae of Strongyloides were easier to detect in saline mounts and as they were rendered non-motile by iodine and LPCB, it was relatively difficult to identify them in the latter.

While wet mounts could detect 10 (only 47.62%) *Isospora* oocysts, MKAF staining could identify 21. Only MKAF staining could detect *Cryptosporidium* spp. Observations made in the present study suggest that the routine use of combination of wet mounts with MKAF staining and Mod. Trichrome staining to be effective in detecting a variety of enteric parasites, in stool specimen of HIV patients. As many HIV-infected asymptomatic patients are known to harbor enteric parasites, it is better to screen all HIV patients periodically for them, especially when CD4 counts are low.

Formalin Ether sedimentation concentration procedure was employed in the study to concentrate stool specimen. After concentration, sensitivity was slightly increased in wet mounts and the increase was 13.04% in MKAF staining. Another advantage of concentration procedure was that, it reduced fecal debris and made identification of parasites easier.

SUMMARY

Stool samples of randomly selected 100 HIV seropositive subjects (cases) and 50 clinically healthy individuals (control), in Chennai, were examined for enteric parasites. Saline, iodine, LPCB wet mounts; modified Kinyoun's acid-fast staining for coccidian parasites and modified Trichrome for Microsporidia were done. Formalin ether sedimentation technique was followed as concentration procedure and microscopic examination repeated with the sediment obtained. CD4 cell counts were done for HIV infected individuals and were used as indicators of immune status to analyze the results obtained.

Enteric parasites were identified in 33% of cases and 16% of controls. Cases with chronic diarrhoea had higher infection rates (63.33%) than those with acute diarrhoea (31.58%) and those without diarrhoea (15.62%). Protozoa were more common than helminths in cases. Isospora belli was the commonest parasite identified in the cases (21%) followed by E. *histolytica/dispar* (5%), *Strongyloides, Cryptosporidium*, hook worm and *E. coli* (2% each); *Microsporidium spp.* and *Giardia* (1% each). Among controls, helminths outnumbered protozoa. In controls *Ascaris* (6%) was the commonest parasite identified followed by *Trichuris* (5%), hook worm (2%), *E.histolytica/dispar* (2%) and *E.coli* (2%).

CD4 cell counts were found to be lower in cases with diarrhoea (319.93 cells/mm^3) than those without; and lower in those with chronic diarrhoea (179.1 cells/mm^3) than those with acute diarrhoea (239.26 cells/mm^3). Maximum number of opportunistic parasites was seen in cases with CD4 counts between 100-200 cells/mm^3.

All conventional parasites and nearly half of the *Isospora* were detected by wet mounts. All the three wet mounts showed almost similar number of parasites. Modified acid-fast staining could identify maximum number of Isospora and *Cryptosporidium* were seen only by this method. *Microsporidium* was seen in Mod. Trichrome stained smears only. Formalin ether sedimentation concentration procedure increased the detection rates in all the methods of parasite identification.

CONCLUSION

Individuals with HIV/AIDS, because of their compromised immune status are at a higher risk of infections and especially opportunistic enteric intracellular parasites affect the small intestine and produce overwhelming results with grave prognosis. As parasites cause prolonged, life-threatening diarrhoea in AIDS patients, identification of these opportunistic parasites at the earliest will enable the clinician to give effective treatment and save the patient from increasing mortality.

Protozoan parasites were found to be more common than helminths in HIV patients, of which coccidia comprised the major portion. *Isospora* was the commonest parasite detected. In the control group helminths were more common than protozoa and coccidian parasites and *Stongyloides* were not detected.

HIV-infected individuals with lower immunity, as indicated by CD4 counts, suffered more with diarrhea, especially in chronic form. Parasites were common in lower immune status. *Isospora* was detected in a wide range of immune status including those without diarrhea, but highest rate of infection was seen with CD4 counts 100-200 cells/mm^3. In the present study, simple techniques like wet mounts and modified acid fast staining could successfully identify a variety of enteric parasites in HIV patients. Formalin ether concentration procedure increased the sensitivity of detection.

APPENDIX

Saline (0.85% NaCl)[39]:

NaCl……0.85 g

Distilled water…100.00 ml

Dissolve the NaCl in distilled water.Label and sterilize by autoclaving.

D'Antoni's Iodine[39]:

Potassium Iodide……1.0 g

Powdered iodine crystals……...1.5 g

Distilled water…….100.0 ml

1. Dissolve the Potassium iodide and iodine crystals in distilled water.
2. The potassium iodide solution should be saturated with iodine, with some excess crystals left on the bottom of the bottle.
3. Store in a brown, glass-stoppered bottle at room temperature and in the dark.
4. This stock solution is ready for immediate use. The stock solution remains good as long as excess of iodine crystals remain at the bottom of the bottle.
5. Aliquot some of the iodine into a brown dropper bottle. The working solution should have a strong tea color and should be discarded when the color lighten (usually within 10 to 14 days).

Note: The stock and working solution formulas are identical, but the stock solution is held in the dark and will retain the strong-tea color while the working solution will fade and have to be periodically replaced.

Lactophenol Cotton Blue Solution[12]

Phenol crystals……....20.0g

Lactic acid……20.0ml

Glycerol……40.0ml

Distilled water……..20.0ml

Cotton blue (or methyl blue)…..0.075g

Dissolve the phenol crystals in the liquids by gentle warming and then add the dye.

Kinyoun's Modified Acid-fast staining[39]

Reagents:

1. 50% Ethanol

   Add 50 ml of absolute ethanol to 50ml of distilled water.

2. Kinyoun's Carbol Fuchsin

   Dissolve 4 g of basic fuchsin in 20 ml of 95% ethanol (solution A).

   Dissolve 8 g of phenol crystals in 100 ml of distilled water.

   Mix solution A and B.

3. 1% Sulfuric Acid

   Add 1 ml of conc. Sulfuric acid to 99 ml of distilled water.

4. Loeffler's Alkaline Methylene Blue

   Dissolve 0.3 g of methylene blue in 30 ml of 95% of ethanol.

   Add 100 ml of dilute (0.01%) potassium hydroxide.

   All these reagents can be stored at room temperature. They are stable for one year.

Formalin-Ethyl Acetate Sedimentation Concentration Technique[39]

Reagents:

5 or 10% Formalin can be used.

Formalin Conc.	Formaldehyde (USP)	0.85% NaCl
5%	50 ml	950 ml
10%	100 ml	1900 ml

Note: Formaldehyde is normally purchased as a 37 to 40% solution; however, for dilution, it should be considered to be 100%. Distilled water may be used in place of normal saline.

Modified Trichrome Stain (Weber-Green modification for Microsporidia)[39]

1.Chromotrope Stain

Chromotrope 2R……..6.0g

Fast green FCF……....0.15g

Phosphotungstic acid……..0.7g

Acetic acid (glacial)…….3.0ml

Distilled water…….100.0ml

1. Prepare the stain by adding 3 ml of glacial acetic acid to the dry ingredients. Allow the mixture to stand (ripen) for 30 min at room temperature.
2. Add 100ml of distilled water. Properly prepared stain is dark purple. Store at room temperature. The shelf life is at least 24 months.

2.Acid-Alcohol

90% Ethyl alcohol …….995.5ml

Acetic acid (glacial)…….....4.5ml

Prepare by combining the two solutions.

Agar for Agar Plate Culture for Strongyloides[39]

Agar……1.5%

Meat extract……0.5%

Peptone…...……1.0%

NaCl……..0.5%

Note: Positive tracking on agar plates has been seen on a number of different types of agar. However, the most appropriate agar formula is that listed above.

PROFORMA

1. Name
2. Patient ID No.:
3. Age
4. Sex
5. Address
6. Religion
7. Occupation
8. Socio-economic status:
9. Literacy: Literate/ Illiterate:
10. History of present illness:

I. Presenting complaints

- Diarrhoea – Yes/No : If yes,
  Frequency - /day
  Duration –
  No. of episodes –
  Consistency of stool –
  Others – Blood/ Mucus/ Pus/ Frothy
- Other gastrointestinal symptoms – pain abdomen/ nausea/ vomiting/ others
- Weight loss: < 10% > 10%
  Duration
- Fever: Duration: < 1 month > 1 month
  Type of fever -
- Other symptoms

II. Past history

History of tuberculosis - Yes / No
History of anti-tubercular treatment
Antiretroviral therapy

III. Family history

IV. Personal history: Married/ Unmarried

A) History of exposure
B) History of blood transfusion
C) History of IV drug abuse
D) History of occupational exposure

V. Clinical Examination

a) General Physical Examination

b) Systemic examination

CVS:
RS:
PA:
CNS:

VI. Clinical Diagnosis

VII. Investigations

A. HIV antibodies detection

a) HIV EIA Comb
b) ELISA
c) HIV Bidot
d) Tri Dot

B. Stool examination

1) Macroscopic examination

- Colour
- Consistency
- Blood / Mucous
- Presence of segments

2) Microscopic examination

(i) Saline Wet Mount

Before concentration –
After concentration -

(ii) Iodine Wet Mount

Before concentration –
After concentration -

(iii) Modified Ziehl-Neelsen staining

Before concentration –
After concentration -

(iv) Modified Trichrome staining

Before concentration –
After concentration -

3) CD_4 cells/mm^3

REPORT

Date: **SIGNATURE**

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KEY TO MASTERCHART

AC	--	After Concentration
ASC	--	*Ascaris lumbricoides*
BC	--	Before Concentration
CD4	--	CD4 T lymphocyte count (cells/mm^3)
CRY	--	*Cryptosporidium spp.*
EH	--	*Entamoeba histolytica/dispar*
EC	--	*Entamoeba coli*
F	--	Female
GIA	--	*Giardia lamblia*
ISO	--	*Isospora belli*
IWM	--	Iodine Wet Mount
LCBWM	--	Lacto-phenol Cotton blue Wet Mount
M	--	Male
MIC	--	*Microsporidium spp.*
Mod AFS	--	Modified Acid-Fast Staining
Mod Tri	--	Modified Trichrome Staining
STR	--	*Strongyloides stercoralis*
SWM	--	Saline Wet Mount
TT	--	*Trichuris trichura*
HW	--	*Hook worm*